



Human erythroid cells are affected by aluminium. Alteration of membrane band 3 protein

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Abstract

There is evidence that anaemia is associated with aluminium (Al). We have already reported on the sensitivity to Al, showed by erythroid cell populations of animals chronically exposed to the metal. In order to investigate whether Al could also affect human cells, experiments were carried out both on immature and mature human erythroid cells. Erythroid progenitors (CFU-E, colony-forming units-erythroid) concentrated from human peripheral blood were cultured in an Al-rich medium under erythropoietin stimulation and their development analysed. Human peripheral erythrocytes were aged in the presence of Al. Cells were examined using scanning electron microscopy, and membrane proteins analysed by polyacrylamide gel electrophoresis with sodium dodecyl sulphate and immunoblotting. The development of the Al-treated progenitors was 8750/6600–9200 CFU-E/ 10^6 cells, a significantly lower median value ($P < 0.05$) than that showed by non-treated cells (12 300/11 200–20 700 CFU-E/ 10^6 cells). Erythrocyte morphological changes were induced by Al during the in vitro ageing. The cells lost their typical biconcave shape, turning into acanthocytes and stomatocytes. Simultaneously, an increased membrane protein breakdown compatible with band 3 degradation was detected. Besides, Al was found within the cells and attached to the membrane. The present in vitro results suggest that Al may disturb human erythropoiesis through combined effects on mature erythrocytes and cellular metabolism in late erythroid progenitors. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Aluminium (Al) loading, as a contributing factor for the aggravation of the anaemia observed in the final stage of renal disease, was first noticed in patients under dialytic treatment and ingesting Al-containing phosphate binders [1–4]. Considering the

multiple factors affecting erythropoiesis in chronic renal disease, the use of experimental animals without renal disorder better established the linkage between Al and the development of anaemia. Regardless of the Al compound and the administration route, anaemia has been induced in rabbits, mice and rats [5–10]. The in vivo effect of Al showed a steep reduction in erythroid progenitor cell growth. Really, this inhibition seemed to be the main early feature of chronic Al-overloading [8–11].

Another important issue should be considered with regard to the action of Al on erythropoiesis. The

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deleterious effect of Al could not be restricted merely to the erythroid progenitors since mature erythrocytes might also be affected. The fact that Al induces changes in red blood cell (RBC) morphology has been established in Al-overloaded rabbits by the finding of anisocytosis (abnormal cell size variations) and poikilocytosis (abnormal cell shape variations) in blood smears [5]. Similar results were found in rats chronically treated with Al citrate [10]. Moreover, electron microscopy analysis of RBCs revealed the loss of their typical biconcave shapes, and the appearance of anomalous cells such as leptocytes (thin and plain), acanthocytes or echinocytes (spiculated) and target cells, strongly suggesting membrane alterations [10].

Not much information is available regarding the effects of Al on human erythrocytes. In vitro short-term assays showed that the highly lipophilic Al acetylacetonate interacts with the membrane of human erythrocytes, resulting in alterations of their morphology from discoid to both echinocytic and stomatocytic forms [12].

In normal red cells, the biconcave disc shape creates an advantageous surface area–volume relationship, allowing red cells to undergo marked deformation while keeping a constant surface area. Directly or indirectly, membrane integral and skeletal proteins – band 3, actin and spectrin – as well as protein–protein interaction play an important role in the regulation of factors that influence cellular deformability [13]. Regarding this subject, one of the aims of the present investigation was to study the effects of Al compounds on the morphology of human erythrocytes and on the integrity of their membrane proteins. With this purpose, the ability of Al to modify human RBCs treated for different periods was evaluated by scanning electron microscopy and immunoblotting. Finally, in order to determine the effects of the metal on immature human erythroid cells, the development of erythroid progenitors grown in an Al-rich medium was studied.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade.

α -Minimum essential medium (α -MEM), 199 medium, penicillin–streptomycin, HEPES buffer, bovine serum albumin (BSA), human apo-transferrin (apo-Tf), *p*-nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP), 2,7-diaminofluorene (DAF), alkaline phosphatase-conjugated antibody to mouse IgG, monoclonal anti-human band 3 (clone BIII-136), monoclonal anti-actin (clone AC-40), and monoclonal anti-spectrin α and β (clone SB-SP1) were purchased from Sigma, USA. Rainbow molecular weight marker and Ficoll-Paque Plus were from Amersham Pharmacia Biotech, Sweden. Silver Stain Kit was from Bio-Rad, USA. Foetal bovine serum (FBS) was obtained from Bioser, Nutrientes Celulares S.A., Argentina, and recombinant human erythropoietin (Epo) Pronivel, from Elea S.A.C.I.F. y A., Argentina.

Ultrapure water with 18 M Ω specific resistivity (Simplicity 185, Millipore, USA) was employed to prepare all solutions and media.

2.2. Colony assay of human peripheral erythroid progenitors

Erythroid progenitor cells concentrated from human peripheral blood were separated over Ficoll-Paque Plus (1.077 g/cm³) by centrifugation at 400 \times *g* for 30 min at 25°C. Interface mononuclear cells were collected and washed twice with α -MEM containing 2% FBS. In order to separate adherent from nonadherent cells, mononuclear cells were suspended in α -MEM containing 50 mg/l gentamicine sulphate and 30% FBS at a concentration of approximately 5 \times 10⁶ cells/ml. Adherent cells were removed by an overnight incubation in culture dishes at 37°C in a 5% CO₂ atmosphere.

Triplicate cultures were carried out in semisolid medium following the technique described by Garbossa et al. [14] with modifications. Briefly, non-adherent mononuclear cells were plated at a concentration of 3.5 \times 10⁵/ml in a mixture containing α -MEM, 0.8% methylcellulose, 25 mM HEPES buffer, 26 mM NaHCO₃, 30% FBS, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 50 mg/l gentamicine sulphate, 1 μ M apo-Tf and 2 IU/ml Epo. Two parallel series were performed in the presence (Al) or absence (C) of 100 μ M Al citrate. Cultures were

incubated at 37°C for 10 days in an atmosphere containing 5% CO₂ and 100% humidity. Small erythroid colonies consisting of eight or more cells, with at least two haemoglobinised cells, were counted as colony-forming units-erythroid (CFU-E). These colonies were stained with 0.9 mg/ml DAF, 0.2% H₂O₂ in 0.2 M Tris-HCl buffer, pH 7.0 [15], and counted using an inverted microscope. Geometric means of triplicates were calculated for each assay. Cultures without Epo were routinely included.

2.3. *In vitro* erythrocyte ageing

Heparinised human peripheral blood was collected from laboratory staff members (29–35 years old). Plasma and buffy coat were carefully removed after 10 min centrifugation at 500×g and 4°C. RBCs were harvested after repeated washing in 10 volumes of isotonic phosphate-buffered saline (0.137 M NaCl, 3 mM KCl, 1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4).

Packed cells were suspended in 199 medium, containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and subjected to *in vitro* ageing for different periods (from 3 to 14 days).

Two parallel assays were run: erythrocytes aged for 't' days without Al (C_t) and erythrocytes incubated for the same period in the presence of 10 or 100 µM Al concentrations, added as either Al citrate or Al chloride (Al_t).

Medium renewal with or without Al was performed every 2 days. Fresh erythrocytes were also assayed as control for the ageing process (C₀).

2.4. Ghost preparation

The whole procedure was performed at 4°C. Erythrocytes were haemolysed by diluting 1:10 with hypotonic 5 mM sodium phosphate buffer, pH 8.0, containing 50 µM phenylmethylsulfonyl fluoride, 50 mg/l soybean trypsin inhibitor, and 1 mg/l aprotinin. RBC ghosts and supernatant cytosol fractions were obtained after 30 min centrifugation at 13 000×g. Pelleted membranes were repeatedly washed until creamy white. Ghosts were suspended in the lysis buffer. The suspensions and cytosol fractions were kept at –20°C.

2.5. Electrophoresis of membrane proteins

Polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS-PAGE) was carried out employing the discontinuous buffer system of Laemmli [16]. The monomer concentration (T%) in the slab mini gels was 4% in the stacking and 8% in the running gel. Samples were dissociated by mixing them in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and bromophenol blue as tracking dye) and then boiled for 1 min. Amounts of 5 µg of protein were applied in each lane. Protein concentrations had been previously determined by the method developed by Lowry et al. [17]. Constant current of 20 mA during the isotachophoretic process in the stacking gel and 25 mA throughout the running gel were applied; the whole electrophoretic procedure lasting approximately 1 h. A molecular weight protein standard was included in each slab gel. At the end of the running, a double staining using silver reagent and Coomassie brilliant blue R-250 (CBB) was developed according to Dzandu et al. [18]. Gels were first stained with Bio-Rad silver kit and then with 0.2% CBB in 30% methanol/10% acetic acid, allowing a colour-coded differentiation of proteins.

Gels run in parallel were used for immunoblotting.

2.6. Immunoblot technique

Western blot was carried out following the methodology described by Towbin et al. [19]. Proteins were electrotransferred to nitrocellulose membranes for 1.5 h employing transfer buffer (25 mM Tris, 195 mM glycine, 0.05% SDS, pH 8.3, 20% (v/v) methanol). Blots were soaked overnight in 2% BSA in TBS (25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4), incubated with primary antibodies for 1 h and washed twice with TBS–0.05% Tween and once with TBS. Incubation with alkaline phosphatase-conjugated antibody to mouse IgG as a secondary antibody was performed for 1 h. Unbound proteins were removed by repeating the above mentioned washing procedure. Then, BCIP and NBT reagents were used as substrate and colour developer, respectively. Monoclonal anti-human band 3, anti-actin, and anti-spectrin were used as first antibodies. Appropriate dilutions were carried out in 1% BSA in TBS.

2.7. Scanning electron microscopy

One drop of each erythrocyte suspension sample was placed on a 50 mm² glass support and fixed for 20 min by 3% glutardialdehyde in 0.1 M phosphate buffer, pH 7.4. Samples were washed three times with the phosphate buffer, and subsequently dehydrated by successive washing in graded acetone (from 25 to 100%). The critical drying step was performed in a Balzers CPD 030 Critical Point Bomb using carbon dioxide as transition fluid. Samples were coated with a thin layer of gold-palladium (Balzers Union SCD 040) and examined with a scanning electron microscope (Phillips 515).

2.8. Aluminium content in cellular fractions

In order to avoid Al trace contamination, all the plasticware used was immersed in 30% HCl for 1–2 h and then exhaustively rinsed with ultrapure water. Al levels were measured in erythrocyte fractions, Al working solutions, culture media and ultrapure water using an atomic absorption spectrometer (Shimadzu AA-6501, Japan) coupled to a graphite furnace atomiser (Shimadzu GFA-6000, Japan) with auto-sampler.

The Al content, determined in membrane and cytosol fractions after erythrocyte lysis, was expressed per protein concentration, which was measured by the Lowry method [17].

2.9. Statistical analysis

The results are expressed either as mean and standard error or as median and range. Comparisons between groups were carried out by the non-parametric Mann–Whitney U-test, and least significant differences with $P < 0.05$ were considered the criterion of statistical significance.

3. Results

3.1. Effect of Al on erythroid progenitor cell development

In order to elucidate if Al has any effect on erythroid differentiation of human immature cells, ery-

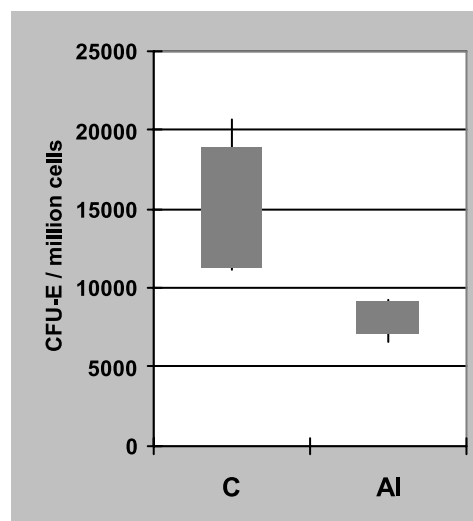


Fig. 1. Effect of Al on erythroid progenitor cell development. Human erythroid progenitors concentrated from peripheral blood were stimulated in vitro by Epo, and cultured in the presence (Al) or absence (C) of 100 μM Al citrate for 10 days. The figure shows the significant 30% decrease of the CFU-E number with respect to controls when erythroid progenitor cells were grown in contact with Al ($n = 5$, $P < 0.05$).

throid progenitors concentrated from peripheral blood were cultured in the presence or absence of Al citrate under Epo stimulation. As can be seen in Fig. 1, the addition of Al to the culture medium induced a significant 30% decrease in the development of erythroid colonies with respect to controls. Results of five assays expressed as median/range, were C: 12300/11200–20700 and Al: 8750/6600–9200 CFU-E/ 10^6 cells ($P < 0.05$). Box plots represent interquartile range, including data between 25th and 75th percentiles. Vertical bars indicate the minimum and maximum values.

3.2. Changes in cell morphology

The effect of Al on cell morphology was studied by scanning electron microscopy of erythrocytes that had been incubated in the presence of either Al citrate or Al chloride for 14 days. The ageing process slightly affected the erythrocyte shape. Except for a few crenated cells, none of the samples aged without Al showed altered cells (Fig. 2a). On the other hand, erythrocytes incubated with Al showed many abnormal shapes (Fig. 2b–f). The typical erythrocyte biconcave shape turned into acanthocytic (Fig. 2c,e)

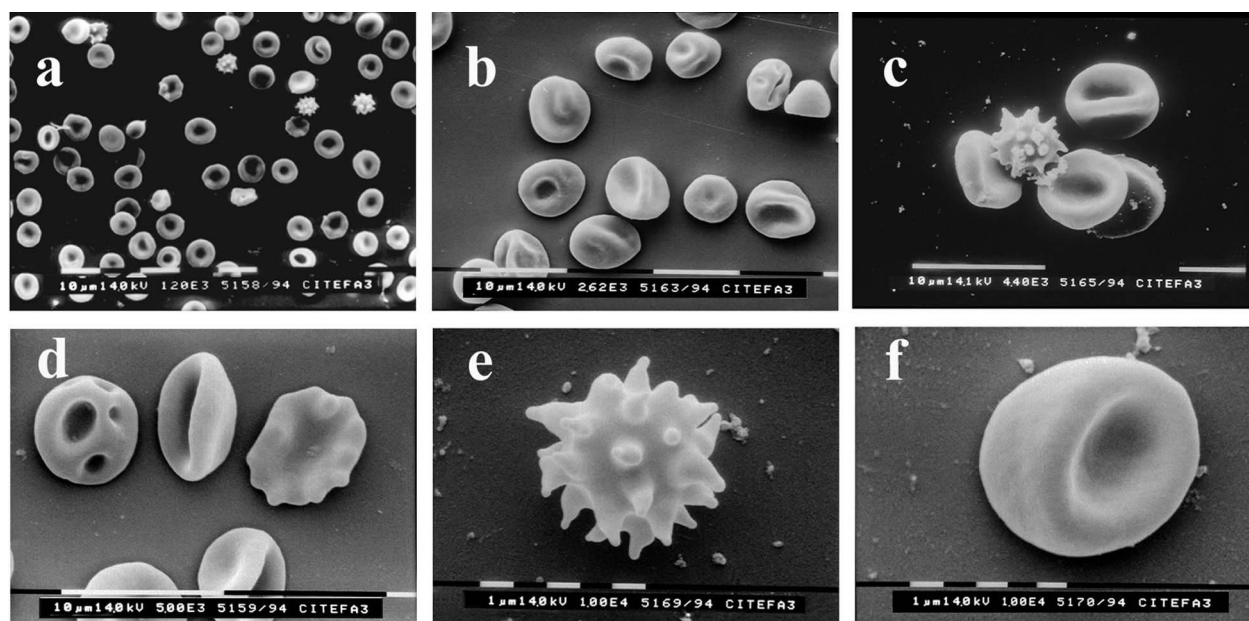


Fig. 2. Scanning electron microscopy of human erythrocytes aged in vitro for 14 days in the absence (a) or presence (b–f) of Al. (a) General aspect of a control sample of aged erythrocytes (1200 \times). (b) General aspect of Al-treated erythrocytes showing the loss of their normal shape and the presence of stomatocytes (2620 \times). (c) Stomatocytic and crenated shapes after cells were incubated with Al (4400 \times). (d) Different abnormal shapes induced by Al (5000 \times). (e) Details of an acanthocyte (10000 \times) and (f) a stomatocyte (10000 \times).

and stomatocytic (Fig. 2b,c,f) shapes. The former, also named echinocyte or crenated cell, is a spiculated cell, while the latter, a cup-shaped cell, displays one important cavity. Both acanthocytes and stomatocytes seem to be the predominant erythrocyte shapes induced by the presence of Al.

These effects produced by Al on RBCs were detected even at low concentrations. Approximately

20–30% of acanthocytes and stomatocytes developed from erythrocytes aged in the presence of 10 μ M Al compounds (data not shown).

3.3. Electrophoresis and immunoblotting

A close relationship can be established between membrane organisation and cell shape. Taking into

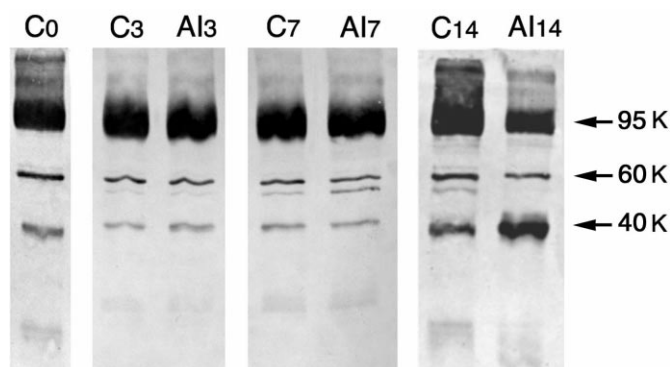


Fig. 3. Profiles of proteins from control and Al-treated RBC membranes. Human erythrocyte membrane proteins were analysed by SDS-PAGE and immunoblot with antibody to band 3. The ghost proteins were obtained from: fresh RBCs (C₀), RBCs incubated for 3 (C₃), 7 (C₇) and 14 (C₁₄) days, and RBCs incubated in the presence of 100 μ M Al chloride for 3 (Al₃), 7 (Al₇) and 14 (Al₁₄) days. Patterns of band 3 protein from erythrocytes aged in the presence of Al show a decreased concentration of the complete molecule of band 3 ($M_r \approx 95000$) and increased amounts of band 3 fragments of lower M_r (≈ 40000). Studies performed at 3, 7 and 14 days suggest a time-related increasing of band 3 degradation products.

account the induction of altered erythrocyte shapes due to Al, the following step was to analyse membrane proteins from erythrocyte ghosts.

Membranes obtained from RBCs incubated with Al for 3, 7, or 14 days were solubilised and analysed by SDS-PAGE. The protein patterns in the double-stained (silver plus CBB) gels showed hardly any difference between samples aged in the presence or absence of Al.

In order to detect individual proteins and their possible minor fractions, an immunoblotting technique employing monoclonal antibodies to band 3, spectrin, and actin was applied in parallel gels.

Figure 3 shows the characteristic immunoblots of membrane proteins from controls and Al-treated erythrocytes that have been developed with anti-human band 3 antibody. It can be noticed that this antibody normally recognises band 3 protein of relative molecular mass $M_r \approx 95\,000$ and several peptides of lower M_r , migrating in the regions of $M_r \approx 60\,000$, $40\,000$ and $20\,000$. After the ageing of human erythrocytes in an Al-rich medium, immunoblot analysis showed altered patterns of band 3 membrane protein (Fig. 3, Al lanes) when compared to the corresponding control (Fig. 3, C lanes). An increased concentration of fragments of lower M_r , particularly the one of $40\,000$, with diminution of the complete molecule ($95\,000$ fraction) is compatible with a band 3 degradation pattern. The increase of protein degradation along the incubation time, particularly observed at 7 and 14 days, suggests a time-related toxic effect of Al. This pattern describing protein breakdown was specially related to band 3, while no significant changes were observed in immunoblots obtained with antibodies against the proteins actin and spectrin.

3.4. Control assays

Control assays performed in the presence of $100\ \mu\text{M}$ sodium citrate instead of Al citrate showed no changes in the morphology and membrane proteins of the RBCs, suggesting that the alterations observed can not be attributed to osmolarity variations due to Al addition. Moreover, no changes in the pH of the incubation medium were registered throughout experimental periods.

The alterations mentioned above were unrelated to the Al compound used, either chloride or citrate.

3.5. Aluminium concentration in cell membrane and cytosol fractions

In order to find out whether Al was able to enter the cell under the experimental conditions employed

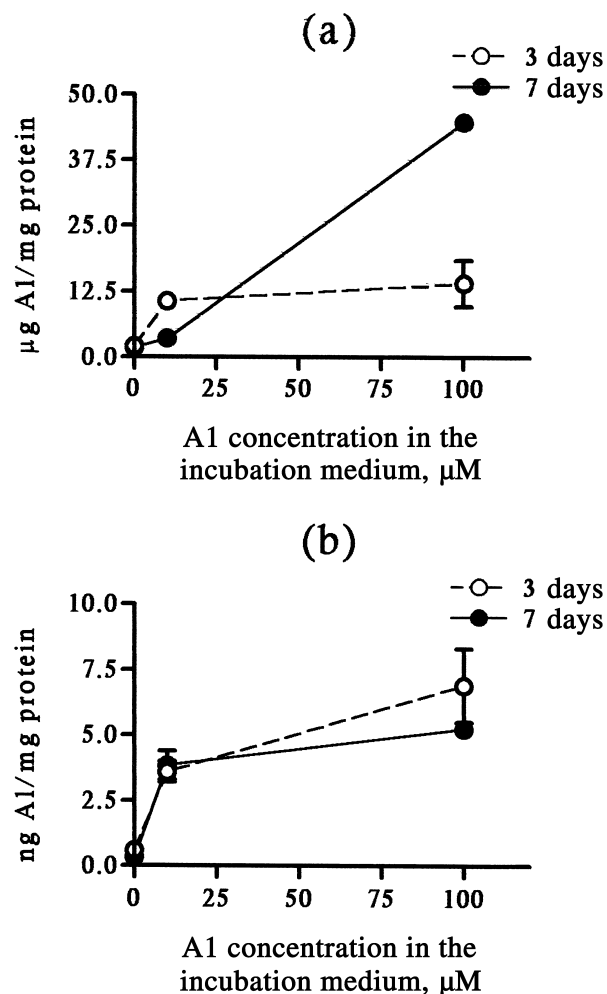


Fig. 4. Al content in erythrocyte fractions. RBCs were aged without Al (control) or in the presence of 10 and $100\ \mu\text{M}$ Al chloride for 3 or 7 days. Al content was measured after RBC lysis in (a) membrane and (b) cytosol fractions. These data are plotted vs. Al concentration in the incubation medium. Each point represents mean \pm standard error of three assays. Al enters the cell (b) and significantly high Al amounts ($P < 0.01$) are found attached to the membrane after long-term incubation with the highest Al dose (a). No significant differences were found when comparing intracellular Al content with either Al doses or incubation time (b).

and, eventually, to study metal distribution within the cell, Al concentrations were determined in membrane (Fig. 4a) and cytosol (Fig. 4b) fractions after erythrocyte lysis. RBCs were incubated in the presence of either 10 or 100 μM Al chloride for 3 and 7 days. Figure 4 shows that Al does enter the cell as it was found in the cytosol fraction (Fig. 4b). Whereas no differences could be observed in the intracellular content in relation to either the duration of the incubation period or the metal dose (Fig. 4b), high amounts of Al were found attached to the membrane after 7-day incubation with 100 μM Al (Fig. 4a). This result was significantly different ($P < 0.01$) from those observed with lower Al doses and through short-term incubation.

The Al content in control fractions which had been incubated throughout the time of the experiments without addition of Al is shown in Fig. 4 at the abscissa value $x = 0$.

4. Discussion

This work shows that human erythroid cells are affected by Al, suggesting that these cells are as sensitive to Al as those from animals and cellular lines.

The 30% decrease of human CFU-E growth observed in cultures developed in the presence of Al (Fig. 1) is in close agreement with the CFU-E inhibition detected when testing bone marrow cells from Al-overloaded animals [8–10]. The fact that the effect observed in these progenitor cells is a depressed response to Epo suggests that the inhibitory effect induced by Al might involve the interaction of the hormone with its target cell. Indeed, we have demonstrated that the *in vitro* inhibitory process induced by Al on mice CFU-E growth was closely related to the presence of Epo, occurred only at early stages of the differentiation process induced by the hormone, and could not be reversed by increasing doses of Epo [14].

The present experimental design focused attention on mechanisms by which Al might affect the erythropoietic system, hence the effects were achieved under high Al exposure.

The alteration of erythrocyte morphology in chronically Al-overloaded rats triggers some questions: Do altered erythroid progenitor cells turn

into abnormal erythrocytes? Or, on the other hand, are mature RBCs affected by direct contact with Al in the blood stream during their life span? In order to further clarify this subject, we evaluated the *in vitro* effects of Al on peripheral erythrocytes by studying not only their morphology but also their membrane components. The results obtained let us assume that Al does affect human erythrocytes in a way that resembles the toxic effects induced in rats by the ingestion of Al [10]. The incubation of erythrocytes with Al was effective to induce changes in their typical morphology, turning them into acanthocytes and stomatocytes (Fig. 2). This observation is in agreement with similar previous findings detected in human [12] and animal [5,10,20] RBCs under the effects of Al.

Certain RBC membrane disorders resulting in the development of abnormal shapes and altered geometry of erythrocytes involve unusual interactions among membrane components. Moreover, several band 3 variants have been associated with acanthocytosis. In the RBC membrane, the bilayer of amphiphilic lipid is anchored to a network of skeletal proteins through transmembrane proteins. Band 3, the major integral membrane protein, interacts with the erythrocyte membrane skeleton that mainly includes spectrin, actin, ankyrin and protein 4.1. The organisation of this particular protein network in the inner surface of the plasma membrane is responsible for maintaining the shape, stability and deformability of erythrocytes [21]. In this work, Al induced membrane protein changes that seem to be restricted to band 3. After the ageing of human erythrocytes in an Al-rich medium, the immunoblot analysis demonstrated an altered membrane protein pattern compatible with band 3 degradation. A decreased concentration of complete band 3 molecule and increased concentration of band 3 fragments of lower M_r appear to be directly related to the duration of the incubation period with Al (Fig. 3). It has been proposed that degradation of band 3 protein is involved in the generation of a senescence signal of erythrocytes that leads to the recognition and removal of old erythrocytes from the circulation [22]. The exact mechanism of this *in vivo* protein degradation during erythrocyte ageing has not been clarified yet, but degradation of band 3 by calpain is known to be increased in RBC membranes of old people as com-

pared with those of young people [23]. Since enhanced calpain activity was found in cerebral cortex of rats following Al exposure [24], a protease-mediated mechanism might be also postulated for the action of Al upon RBC membrane. This mechanism would not exclude the possibility of an effect of Al on the arrangement of the lipidic bilayer as it has been suggested that the bilayer of normal RBC membrane is stabilised by band 3 interactions with membrane lipids [25]. Therefore, according to the bilayer hypothesis in erythrocytes, shape changes induced by foreign molecules may be due to differential expansion of their two monolayers. Spiculated shapes (echinocytes or acanthocytes) may arise when the added compound accumulates in the outer monolayer, whereas cup shapes (stomatocytes) would be observed when the compound is inserted in the inner monolayer [26]. The fact that Al induces both acanthocytes and stomatocytes, such as it was found in this work and in the study by Suwalsky et al. [12], probably indicates that the metal interacts with both the outer and the inner monolayers of the erythrocyte membrane. Even though no specific Al location could be established in the two halves of the bilayer, it is clear that the metal enters the cell and attaches in high amounts to the membrane (Fig. 4). This last observation strongly supports a hypothesis of the formation of membrane gaps due to Al with subsequent membrane re-arrangements [27].

In view of the present in vitro assays, it would be right to assume that RBCs are sensitive to the action of Al. Nevertheless, this would not be an exclusive effect since altered mature erythrocytes might, alternatively, be derived from abnormal progenitors. Therefore, these results suggest that Al may disturb human erythropoiesis through combined effects on mature erythrocytes and cellular metabolism in late erythroid progenitors.

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